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Swiss Expert Committee for Biosafety SECB

Recommendation of the SECB

on the classification of work with genetically modified viral vectors

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1 Purpose and field of application

Viral vectors are very commonly used in biomedical research laboratories. The viruses from which the vectors are derived are generally assigned to group 2, and some are assigned to group 3. Following modification, work with most vectors can be assigned to class 2 and in some cases class 1. This recommendation discusses the classification of work with a number of commonly used viral vectors. Also for other viral vectors a similar risk assessment should be performed.

2 Criteria for risk assessment

For the risk assessment of work with viral vectors, a number of factors have to be taken into account:

- The nature of the transgene (insert) has to be considered (e.g. oncogenic potential, cytokine-encoding sequences). For more details, see the corresponding SECB recommendation on risk assessment of activities with oncogenic and cytokine-encoding sequences, (<https://www.efbs.admin.ch/en/recommendations/recommendations-of-the-secb/>).
- The degree of deletion of the vector.
- The potential of the vector system to regenerate a replication-competent virus from vector components. The use of multi-component systems is an advantage, i.e. the various complementing sequences (helper sequences) necessary for producing the replication-incompetent vector are integrated separately into the cellular genome, or are on two or more plasmids, so that the risk of recombination is lower.
- The titer and the total amount of vector.
- Whether a biologically safe system is used (e.g. pBR322 plasmid backbone or a derivative of it, replication of plasmid only in *E. coli* K12).
- Host- and tissue-specificity of the vector (tropism): can the vector replicate in many different cell types including human and animal cells?
- Work with animals: is the host permissive or non-permissive? Is the animal engrafted with human cells, or transgenic, e.g. carrying human receptors?
- Use of self-inactivating vectors.
- The possibility of activating cellular oncogenes (e.g. through integration of the viral genome into the host genome).
- The group to which the packaging or complementing cell lines belong that provide the helper functions and the target cells used for transduction. For more details, see the corresponding SECB recommendation on the safe handling of human and animal cells and cell cultures (<https://www.efbs.admin.ch/en/recommendations/recommendations-of-the-secb/>).
- The possibility to downgrade virally infected cells or animals in the absence of shedding of viral vector particles (in particular by testing of cellular supernatants or animal droppings or evidence from literature).
- The identity and "infection-free" status of the host cells to prevent creation of replication-competent, recombinant (retro-)viruses (e.g., q.c. of cell lines, test certificates for absence of pathogens, reverse transcriptase-negative testing).

Site directed mutagenesis: CRISPR-Cas9, which is easy to apply in many different groups of organisms, has become widely used for genetic editing and carries enormous possibilities. However, special attention is required, since properties with increased hazard potential might be generated (e.g. mutation in an oncogene or a tumor suppressor gene).

3 Retroviral vectors

The family of retroviruses (*Retroviridae*) is very diverse, consisting of seven genera affecting humans and animals. The viruses are enveloped and replicate in a host cell through the process of reverse transcription of the positive-sense RNA strand. This is needed to get a double-stranded DNA which is stably integrated into the host genome.

The viral genome is flanked by LTRs (Long Terminal Repeats) containing a transcriptional promoter, an enhancer, a primer binding site, a packaging signal (Ψ), and codes for the *gag* (group specific antigen), *pol* (polymerase) and *env* (envelope) genes.

More complex retroviruses such as the lentiviruses have additional open reading frames coding for a number of regulatory proteins.

Retroviral plasmid vectors, such as the Moloney murine leukemia virus (MoMLV) vector, are usually constructed as shuttle vectors that are generally based on a pBR322 or pBR328 plasmid backbone (necessary for replication and selection in bacteria), containing the viral packaging signal Ψ , the primer binding site, the retroviral 5' and 3' LTR, the inserted gene, and possibly a selection marker. Because the packaging signal is deleted in the helper genome, replication-defective viruses are produced when retroviral vector DNA is transfected into packaging cell lines containing the helper genome expressing the viral *env*, *gag* and *pol* genes. This virus can transfect target cells, but is unable to form infectious particles because genes coding for viral proteins are missing. This is a safety measure often used in viral vectors. The viral genes responsible for the replication of the virus are separated from the rest of the genome, thus reducing the risk of a recombination of infectious particles (Figure 3.1).

Packaging cell lines can contain different types of helper genomes. Following transfection with the plasmid vector(s) viral particles with different tropisms will be produced:

ecotropic: host range of murine ecotropic viruses: mice and rat cells

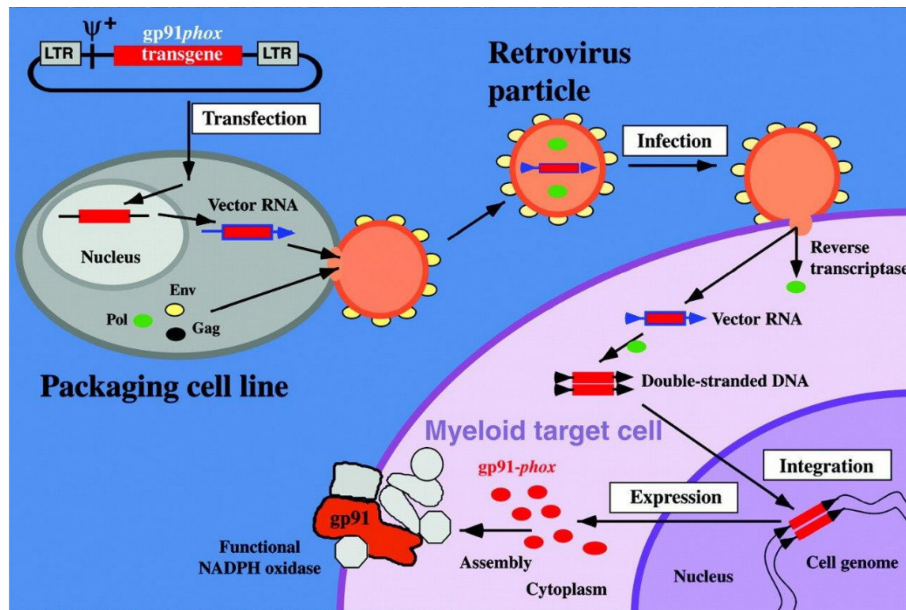
amphotropic: broader host range: murine and non-murine cells, including human cells

pseudotype: envelope proteins are either modified or originate from different viruses, e.g. arenaviruses, alphaviruses, hepadnaviruses, flaviviruses, vesicular stomatitis virus (G-glycoprotein, VSV-G). This can lead to greater stability or a broader host range.

Generally, ecotropic retroviruses are assigned to group 1 while amphotropic and pseudotype retroviruses belong to group 2.

3.1 Lentiviral vectors

Viruses belonging to the family of the more complex *Lentiviridae* are a type of retroviruses and can infect non-dividing and dividing cells (in contrast to the other retroviral vectors, which are only infectious in dividing cells). For this reason, lentiviral vectors are of interest in human gene therapy and great emphasis has been placed on the development of safe vectors. In second and third generation lentiviral vectors several accessory genes have been deleted and 3- or 4-plasmid systems are used for transfection in order to avoid the creation of replication-competent revertants. Newer constructs are inducible by tetracycline or ecdysone, or have tissue-specific promoters.



From: *News Physiol Sci*; Vol.17; June 2002; www.nips.org

Classification:

1. *E. coli* (group 1) with retroviral vector (RV) -> GMO: group/class 1
2. Established cell line (group 1) with RV -> GMO: group/class 1
3. Complementing packaging cell line (group 1) with RV
 - > GMO: ecotropic group/class 1
 - > GMO: amphotropic group/class 2
 - > GMO: pseudotype group/class 2
4. Established cell line (group 1) with transgene
 - > GMO: ecotropic group/class 1
 - > GMO: amphotropic group/class 2
 - > GMO: pseudotype group/class 2
 - No shedding any more group/class 1
5. Primary cells (group 2) with transgene -> GMO: group/class 2

Figure 3.1: Retroviral expression vectors

Classification of work with retroviral and lentiviral vectors

Vector, activity	Group/class
Vector DNA in <i>E. coli</i>	
Introduction of retroviral or lentiviral vector DNA in <i>E. coli</i> K12 or derivatives (production, amplification of vector)	1
Introduction of envelope plasmid (coding for <i>env</i>) or packaging plasmid (coding for viral capsid elements such as <i>rev</i> , <i>gag</i> , <i>pol</i> , etc.) in <i>E. coli</i> K12 or derivatives for the production, amplification of corresponding plasmids	1
Murine ecotropic retroviruses	
Production of replication-defective ecotropic vectors (insert: group 1) in ecotropic packaging cell line (group 1) through transfection with retroviral vector DNA	1
Recombinant replication-defective ecotropic murine retroviruses, produced by ecotropic packaging cell lines	1
Replication-competent ecotropic murine retroviruses	1
Cell lines (group 1) infected with replication-defective ecotropic retroviruses (not shedding viruses with broader host range)	1
Recombinant replication-defective ecotropic murine retroviruses with an oncogene	1
Murine amphotropic retroviruses	
Packaging cell lines containing only sequences coding for amphotropic envelope	1
Infection of amphotropic packaging cell lines (group 1) with replication-defective ecotropic murine retrovirus	2
Production of replication-defective amphotropic vectors in amphotropic packaging cell line (group 1) through transfection with retroviral vector DNA	2
Transfection of a co-culture of ecotropic and amphotropic packaging cell lines with retroviral vector DNA	2
Recombinant replication-defective murine amphotropic retroviruses shed by amphotropic packaging cell lines	2
Lentiviral vectors	
Transfection of cell lines (group 1) with lentiviral vector DNA and plasmids containing the genes necessary for packaging and the envelope (<i>rev</i> , <i>gag</i> , <i>pol</i> and <i>env</i>)	2
Recombinant replication-defective lentiviral vectors shed by above cell lines	2

Vector, activity	Group/class
Cell lines and primary cells infected with recombinant retroviral or lentiviral vectors	
Cell lines assigned to group 1 and infected with above described group 2 replication-incompetent retroviral vectors	2
If it can be assumed that following several cycles of passaging and washing, the above cells do not shed viral vector particles any more, they can be re- classified to class 1 (as long as they cannot complement the replication defect and are not contaminated with replication-competent retroviruses)	1
Primary human and animal cells are often assigned to group 2 (see SECB recommendation on the safe handling of human and animal cells and cell cultures https://www.efbs.admin.ch/en/recommendations/recommendations-of-the-secb/). They remain assigned to class 2 when infected with replication-incompetent retroviral or lentiviral vectors even when they stop shedding viral	2
Vectors with modified envelope proteins	
Replication-defective retro- and lentiviral vectors containing an envelope protein from ecotropic murine leukemia viruses	1
Infection of group 1 cells with above retro- or lentiviral vectors (if group 2 cells are used, then class 2)	1 (2)
Replication-defective retroviral vectors containing envelope proteins from other viruses, e.g. vesicular stomatitis virus (VSV), Gibbon ape leukemia virus (GaLV), RD114 (feline leukemia virus)	2
Replication-defective lentiviral vectors containing envelope proteins from other viruses, e.g. vesicular stomatitis virus (VSV), Gibbon ape leukemia virus (GaLV), RD114, or recombinant MoMLV envelope proteins containing the ligand for a cellular surface protein	2
Infection of group 1 or 2 cells with above retro- or lentiviral vectors	2
Infection of animals with retroviral or lentiviral vectors	
As long as animals infected with group 2 retro- or lentiviral vectors can shed these viral particles in any form, they must be kept under safety level 2 conditions	2
Once it can be assumed that infected animals no longer shed viral particles, they may be kept at safety level 1. This can be after a relatively short time (see Karlen et al, 2007): In mice infected with 10 ⁷ transfecting units of LacZ lentiviral vector intravenously, no transfecting unit could be detected 24 hours postinjection	1
Lentiviral vectors containing oncogenic sequences: Special attention must be given to pseudotype lentiviral vectors which are more stable, have a host specificity for human epithelial cells, or cannot be recognized by the human complement system due to an altered glycosylation pattern. See the corresponding SECB recommendation on risk assessment of activities with oncogenic and cytokine-encoding sequences, https://www.efbs.admin.ch/en/recommendations/recommendations-of-the-secb/	2 or 3

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4 Adenoviral vectors

Adenoviral vectors used for gene transfer are usually replication defective due to a deletion in the E1 early region. Adenoviruses are assigned to group 2, see *Classification of organisms* <https://www.bafu.admin.ch/bafu/en/home/topics/biotechnology/publications-studies/publications/classification-of-organisms.html>. Many vectors also have a deletion in the early region E3 which is not essential for virus replication (first generation vectors); second generation vectors additionally have deletions in early regions E2 and E4, leading to a defect in late protein synthesis. In order to produce infectious particles, plasmids containing the defective adenovirus genome and the gene to be expressed are introduced into cells constitutively expressing the E1A genes, such as the human 293 cells. The so-called *gutless* vectors retain only the *cis* elements necessary for DNA replication and packaging of the viral DNA. They lack all the adenoviral genes and are therefore helper-virus dependent.

Work with adenoviral vectors is generally assigned to class 2. However, if only individual steps are involved, it is possible to classify some of the steps into class 1. For the risk assessment it is important to consider the possibility that replication competent viral particles can arise from homologous recombination. However, this depends on the helper cell lines used for production of the viral vectors, and the probability is very low when cell lines such as PER.C6 or A549 cells are used. Such well characterized helper cell lines, which only contain short adenoviral sequences have been assigned to group 1. The risk of integration of DNA into the genome depends on the type of adenoviral vector used. It has been shown that only type 12 has a high integration rate and can induce tumour growth in new-born rodents. Adenovirus type 5 vectors do not have any oncogenic potential and are therefore the most commonly used to create new vectors. These vectors are also used in gene therapy and for vaccine production.

Classification of work with adenovirus type 5 vectors

Vector/activity	Group/class
Vector DNA	
Intact adenovirus genome, free or inserted into pBR322-based plasmid, in <i>E.coli</i> K12 or derivatives (production, amplification of intact adenovirus genome)	2
Replication-defective adenovirus genome, free or inserted into pBR-based plasmid, in <i>E.coli</i> K12 or derivatives (production, amplification of replication-defective adenovirus genome)	1
Transfection of group 1 or 2 non-complementing cells with replication-defective adenovirus genome, free or inserted into pBR-based plasmid: classification as for recipient organism	1 or 2

Vector/activity	Group/class
Viral particles	
Complementing packaging cell lines (e.g. 293 cells)	1
Production of replication-competent adenovirus through transfection of group 1 or 2 non-complementing cells with intact adenovirus genome, free or inserted into pBR-based plasmid	2
Production of replication-defective, recombinant adenovirus (with or without insert) through transfection of group 1 complementing cells with replication-defective adenovirus genome, free or inserted into pBR-based plasmid	2
Production of replication-defective, recombinant adenovirus (with or without insert) through transfection of group 1 or 2 cells with several plasmids containing replication-defective subgenomic or subgenic adenovirus sequences, where the possibility exists that replication competent adenovirus virions can arise by recombination	2
Infection of group 1 or 2 target cells (complementing or not) or animals unable to complement the replication defect with the above mentioned replication-defective, recombinant adenovirus	2
Once it can be assumed that target cells (group 1) of infected animals no longer shed viral particles, they may be kept at safety level 1	1
<i>Gutless</i> adenoviral vector particles in which all adenoviral genes are deleted, no transgene with hazard potential is present, which have been produced in helper cell lines, where no homologous recombination between the integrated helper genes and the helper virus is expected, when packaging of helper virus DNA is minimized and the adenoviral vector particles are set free from contaminating helper viruses by density gradient centrifugation	1
Infection of group 1 target cells or animals with the above-mentioned <i>gut-less</i> adenoviral vector particles	1

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5 Adeno-associated viral vectors

Adeno-Associated Viruses (AAV) are non-pathogenic human and animal *Parvoviruses*. AAVs appear to be defective, requiring co-infection with helper viruses, such as adenoviruses or herpes simplex viruses, in order to replicate. AAVs can also replicate in cells that have been put under stress, such as irradiation or treatment with genotoxic agents, suggesting that they are not fully defective but are rather reliant upon certain cellular conditions for replication.

Many different serotypes of AAV have been isolated, and the list continues to increase. There are six known human AAV serotypes that appear to be highly prevalent, which are assigned to group 2. All other AAV serotypes (e.g. Avian, Bovine, Canine, Equine and Ovine, resp. AAV serotypes 1, 4, 6, 7, 8, 9, 10 and 11 isolated from monkeys) are also assigned to group 2 since the absence of pathogenicity for humans has not yet been proven. However in the absence of a helper virus and if the transgene does not encode a tumorigenic gene or a toxin the vector can be assigned to class 1.

AAV-virions are small non-enveloped particles that carry a linear single-stranded DNA genome, which is approximately 4.7 kb in size. There are two viral open reading frames, *rep* and *cap*, flanked by T-shaped inverted terminal repeats (ITR), which are important for genome replication, packaging, and integration.

AAV-derived vector systems consist of two plasmids, the vector and the helper plasmids. The only AAV genetic elements that are retained in the vector plasmid are the two ITRs flanking the gene of interest whereas the helper plasmid only contains the two viral sequences *rep* and *cap*. In order to produce recombinant, AAV-derived vector particles, host cells were initially simultaneously co-transfected with the vector and helper plasmids and over-infected with a replication-competent helper virus. However, for most AAV-based vector systems used to date, the viral helper functions can be provided independently from a helper virus. By using a complementing cell line (such as HEK293) which expresses *in trans* the adenoviral E1a and E1b proteins, the helper plasmid only has to have the genes coding for the adenoviral E2a, E4 proteins and viral associated RNA. Recombinant or synthetic AAV constructs of all serotypes produced with such gutless vectors and in which the transgene does not encode either a potentially tumorigenic gene product or a toxin molecule and are produced in absence of a helper virus can therefore be assigned to class 1. Where wild-type adenoviruses or herpes simplex viruses are used to supply the helper functions, the activity has to be assigned to class 2.

The different AAV serotypes can enter a cell by different mechanisms leading to a broad host range. It has been shown in human clinical studies that AAV-2 vectors do not get transmitted into the germ line. After 48 h, vector sequences can no longer be detected in blood and urine. This strongly reduces the possibility of disseminating infectious viral particles. Due to the *gutless* nature of the vectors, insertional mutagenesis has never been observed when using AAV-based vector systems for gene transfer. One major limitation of AAV as gene delivery vehicle however is the relatively small packaging capacity of the system.

Classification of work with adeno-associated viral vectors (independently from serotype used)

Vector/activity	Group/class
Vector / helper DNA	
Recombinant AAV vector DNA (containing only the two viral ITRs) or helper plasmid vector in <i>E.coli</i> K12 or derivatives (production, amplification of vector and helper plasmids)	1
AAV-derived viral particles	
Production of AAV-derived vector particles through co-transfection of a host cell line with the vector and helper plasmids and infection with a replication-competent helper virus	2
Infection of group 1 or 2 target cells or animals with the above-mentioned vector particles	2
Production of helper virus-free, AAV-derived vector particles through co-transfection of a complementing cell line (e.g. HEK293) with vector and helper plasmids	1
Infection of group 1 target cells or animals with the above-mentioned vector particles	1
Infection of group 2 target cells with the above-mentioned vector particles	2
Production of helper virus-free, AAV-derived vector particles containing sequences with hazard potential, such as oncogenes, cytokine-encoding sequences, integrases or defined si/mi/shRNA	2
Infection of group 1 or 2 target cells or animals with the above-mentioned vector particles	2
Once it can be assumed that target cells (group 1) or infected animals no longer shed viral particles, they may be kept at safety level 1	1

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6 Herpes simplex viral vectors

Human Herpes Simplex Viruses type 1 and type 2 (HSV-1 and HSV-2) are a class of large DNA viruses with double-stranded genomes capable of accommodating a large amount of foreign DNA. They have a broad host cell range. The HSV virion consists of four components: envelope (11 glycoproteins), tegument (more than 10 proteins), capsid (7 proteins) and the viral genome (152kb for HSV-1 and 155kb for HSV-2).

The majority of adults are seropositive for HSV (e.g. 50% of the population is infected with HSV-1).

The HSV-based vector systems for gene delivery are unique, compared with others available, because of their high efficiency of gene transfer, stability, extensive host cell range (amphotropic) and potentially large transgene capacity. In addition, the HSV-based vector remains as an extrachromosomal episome, which decreases the likelihood of an insertional mutation in the host genome, thus contributing to better safety.

There are two main strategies for HSV-1 based vectors that are commonly used today:

- recombinant, replication-defective HSV (rHSV) and
- plasmid-derived amplicon vectors (HSV amplicons).

rHSV are helper virus-independent and are generated by deleting or mutating one or more viral genes, whose expression is essential for viral replication. Packaging into recombinant viral particles is accomplished with complementing eukaryotic cell lines that provide the missing viral gene products *in trans*. Despite of their replication deficiency, cytopathic effects are still observed when using rHSV, and this is most likely due to the non-deleted immediate early gene product as a source of toxicity.

The HSV amplicons contain fewer viral genes than the rHSV vectors. They are composed of two virally derived non-coding sequences, the HSV origin of DNA replication *oriS* and the cleavage/packaging signal *pac*, as well as the gene of interest. As such, they are by definition helper virus-dependent. Packaging of amplicon DNA into viral particles initially occurred in a cell line transfected with a replication-defective rHSV and which provided the missing gene products *in trans*, giving rise to a combination of amplicon and helper viral particles (up to 50%) as well as wild-type HSV-1 revertants produced by homologous recombination with the packaging cell DNA. Helper virus-free packaging systems have since been developed. The helper virus genome is for example supplied by a set of five cosmids or bacterial artificial chromosomes, in which the *pac* signals are inactivated by mutation. These five elements and the amplicon DNA are co-transfected into permissive eukaryotic cells. Vector stocks are devoid of helper virus and thus cause negligible cytopathic effects. HSV-1 amplicon particles are replication-defective, do not contain viral coding sequences, do not integrate into the host genome and are only infectious for a single round (single-cycle, abortive infection).

For the risk assessment, it is thus important to know if the replication deficiency is stable and cannot be re-established, and whether HSV-1 helper virus or wild-type HSV-1 could emerge that will produce cytotoxic proteins.

Activities with helper virus-free HSV-1 amplicon particles containing inserts without hazard potential can be done under safety level 1. Work involving RNA interference (e.g. by using si/mi/shRNAs) should be done under safety level 2 as little is known about their modifying properties in cells. The same holds true for sequences having hazard potential (e.g. oncogenes, cytokine-encoding sequences, integrases). In the case that a detailed risk assessment is carried out demonstrating that the used si/mi/shRNA insert has no or a negligible risk, work can also be performed under safety level 1 conditions.

Because of the presence of coding viral sequences and consequently of possible cytopathic effects as well as in regard to their extended tropism, activities with rHSV and helper virus-contaminated amplicon particles should generally be carried out under safety level 2 independently of the inserts used.

Classification of work with herpes simplex virus vectors

Vector/activity	Group/class
Vector / helper DNA	
Replication-defective HSV genome or helper plasmid vectors in <i>E. coli</i> K12 or derivatives (production, amplification of vector and helper plasmids)	1
Plasmid-derived HSV amplicon DNA in <i>E. coli</i> K12 (production, amplification of amplicon DNA)	1
Viral / amplicon particles	
Production of recombinant, replication-defective rHSV through transfection of packaging cell line (cytotoxic effects still possible)	2
Infection of group 1 or 2 target cells or animals with rHSV	2
Production of HSV-1 amplicon particles through co-transfection of a complementing cell line with amplicon DNA and replication-defective rHSV helper virus	2
Infection of group 1 or 2 target cells or animals with the above-mentioned amplicon particles	2
Production of helper virus-free HSV-1 amplicon particles in permissive eukaryotic cell lines (group 1)	1
Infection of group 1 target cells or animals with the above-mentioned amplicon particles	1
Infection of group 2 target cells with the above-mentioned amplicon particles	2
Production of helper virus-free HSV-1 amplicon particles containing sequences with hazard potential, such as oncogenes, cytokine-encoding sequences, integrases or defined si/mi/shRNAs	2
Infection of group 1 or 2 target cells or animals with the above-mentioned amplicon particles	2
Once it can be assumed that target cells (group 1) or infected animals no longer shed viral particles, they may be kept at safety level 1	1

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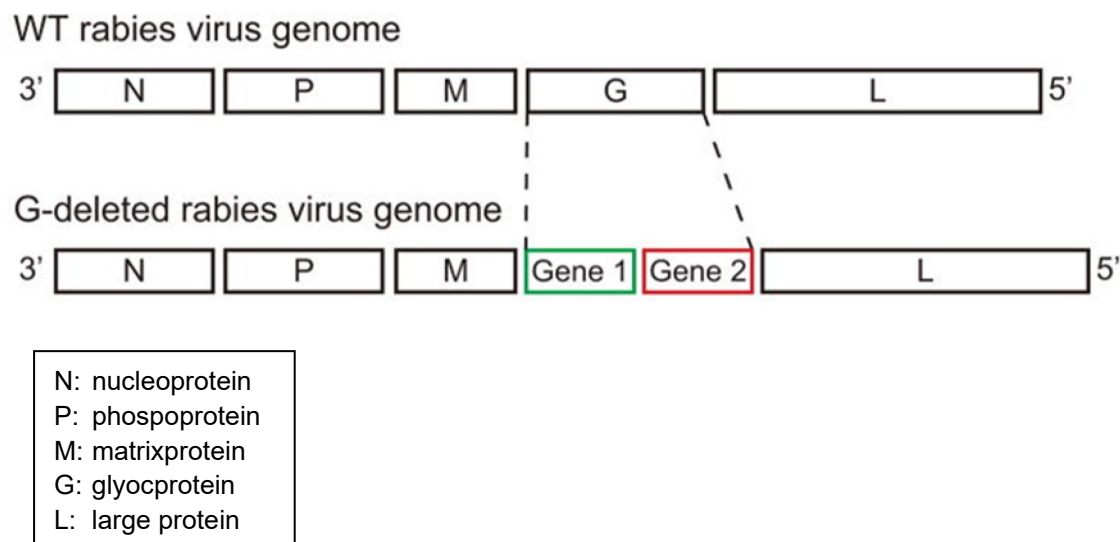
7 Rabies viral vectors

Wild type rabies virus is a single-stranded, negative-sense, enveloped RNA virus belonging to the family of *Rhabdoviridae*. It is a common zoonotic virus that can infect all mammals. Transmission to humans occurs through animal bites. Human to human transmission is not known. In man, with some rare exceptions, it is considered 100% lethal in case no post-exposure prophylaxis (vaccination, immunoglobuline) is initiated before clinical symptoms appear. Rabies infections result in 30'000 to 70'000 death worldwide each year, mostly through bites of infected dogs or cats.

Due to its virulence, rabies is classified as a risk group 3 virus and the Swiss Federal Office of Public Health recommends vaccination of laboratory personnel involved in rabies research. The vaccine strains authorized for human use by Swissmedic are Rabipur (strain Flury LEP) and Mérieux. The vaccines consist of inactivated rabies virus, which has been isolated from infected humans.

Rabies viral vectors used for research are almost exclusively derived from the animal vaccine strain SAD-B19. This attenuated live strain has been used in Europe for vaccination of wildlife via infected bait. It is non-pathogenic for a wide range of animal species. However, certain rodent species still show clinical signs, indicating a low residual pathogenicity of SAD B19. The protein regions of SAD-B19, which represent the linear antigen epitopes, do not have any amino acid sequence difference to Rabipur, indicating that vaccination with Rabipur might provide protective immunity against SAD-B19.

SAD-B19 viral vectors have a deletion in the sequence coding for the G-protein (ΔG) in their RNA genome (Figure 7.1). Upon infection, SAD-B19 ΔG undergoes one complete cycle of intracellular replication and produces progenies that are able to bud from the host cell, even though they are devoid of the G-protein. These SAD-B19 ΔG particles, however, are incapable of attachment and entry into a secondary host cell. Some SAD-B19 ΔG have additional deletions in the N and P genes and the poly-U segments between N and P, resulting in reduced viral protein expression.



Adapted from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4028848/>

Figure 7.1: SAD-B19 viral vectors with deletion of the G-protein (ΔG) in their RNA

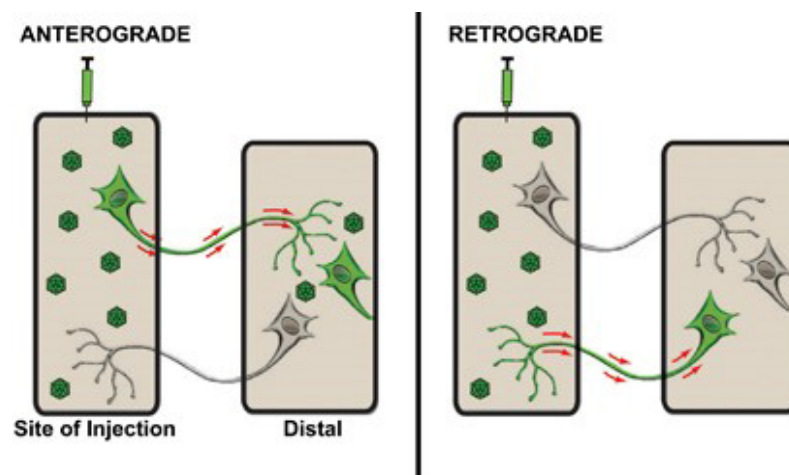
SAD-B19 Δ G is produced by co-transfection of permissive cells with the Δ G-RNA genome plasmid and plasmids coding for the Rabies G-protein. The resulting SAD-B19 Δ G particles with the Rabies G protein in their envelope are able to transduce cells once, but are not able to promote the generation of new infectious viral particles. At no point in the formation of Δ G-Rabies vector (RABV) is intact rabies virus involved. However a recombination cannot be excluded.

The G-protein sequence in the SAD-B19 Δ G genome is either replaced by a sequence coding for a different envelope protein and/or by a sequence coding for a fluorescent marker to allow tracing of neuronal spread after neuron infection. Other variants may code for proteins relevant in neuroscience or vaccine development.

SAD-B19 Δ G, coding for fluorescent markers, is used to trace retrograde mono-synaptic connections between one neuron and the ones directly connected to it (see Figure 7.2). To this aim, SAD-B19 Δ G is injected *in vitro* or *in vivo* into single neurons or a number of specific neurons using the patch-clamp technique.

When neurons are transduced with SAD-B19 Δ G and subsequently co-transduced with a replication-deficient viral vector coding for the rabies G-protein (for instance a vesicular stomatitis viral vector, VSV-RV-G), new SAD-B19 Δ G particles with the G-proteins in their envelope are formed in the transduced neurons. Through this trans-complementation, a secondary trans-synaptic spread of SAD-B19 Δ G to directly connected neurons is possible. Again, as these particles lack the G-protein gene in their genome, formation of infectious particles and further spread is prevented.

For directed transduction of neurons of interest, SAD-B19 Δ G is pseudo-typed with the EnvA glycoprotein from the avian sarcoma and leucosis virus to infect only neurons with the corresponding receptor, TVA. This receptor is absent in mammals and expression in neurons is dependent on transduction with a viral vector (usually AAV) coding for the receptor. In addition to TVA, the vector also codes for the rabies G-protein (AAV-TVA-RB-G). Upon expression of TVA, these cells can subsequently be transduced with pseudo-typed SAD-B19 Δ G-EnvA. Again, this co-transduction results in the formation of new SAD-B19 Δ G particles with the G-proteins in their envelope able for one more round of infection. An additional specification of the EnvA/TVA system can be achieved through the use of a Cre-recombinase dependent helper virus vector (generally also AAV) regulating TVA expression.



From: Salegio, E. A. *et al.*, Gene Ther. 2013 Mar; 20(3): 348–352.

Figure 7.2: Anterograde ('donor' axons) and retrograde ('receptor' axons) axonal trans-synaptic movement of viral vectors

In order to distinguish between retrograde and anterograde connections between neurons, the retrograde tracing methodology using SAD-B19ΔG is combined with an anterograde tracing methodology with specific anterograde-moving strains of herpes simplex virus 1, (HSV-1) or pseudorabies virus (PRV). By using different fluorescent markers (GFP, mCherry) in the respective vectors and co-infection of the neurons, retrograde neuron connections are marked through trans-synaptic spread of SAD-B19ΔG and anterograde connections through trans-synaptic spread of HSV-1 or AAV.

Classification of work with rabies viral vectors

Vector/activity	Group/class
DNA plasmids	
Recombinant G-protein-deleted rabies vector DNA plasmids or helper plasmids containing single rabies open reading frames in <i>E.coli</i> K12 or derivatives (production, amplification of vector plasmid and helper plasmids)	1
Rabies viral vectors in animals or animal cells	
Production of SAD-B19 vaccine strain	2
Production of SAD-B19ΔG particles through co-transduction of a host cell line with the G protein-deleted vector genome plasmid and helper plasmids coding for the G protein and other rabies proteins in trans	2
Co-transduction of group 1 or 2 target cells or co-infection of animals with SAD-B19ΔG and with replication-deficient viral vectors complementing for the rabies G-protein (group 1 or 2, i.e. AAV-RB-G), but no other rabies proteins	2
Production of pseudotyped SAD-B19ΔG-EnvA particles through co-transduction of a host cell line with the G protein-deleted vector genome plasmid and helper plasmids coding for the EnvA glycoprotein and other rabies proteins	1
Transduction of group 1 target cells or infection of animals expressing the TVA receptor with SAD-B19ΔG -EnvA	1
Transduction of group 2 target cells or infection of animals expressing the TVA receptor with SAD-B19ΔG -EnvA	2
Group 1 animals which have been injected with SAD-B19ΔG into the brain (as a class 2 activity), may be downgraded to risk group 1 and kept at safety level 1	1
Analysis of group 1 cells and animal tissues infected with SAD-B19ΔG as long as there is still a chance of infectious rabies vector particles present	2
Analysis of infected primary cells and animal tissues from SPF animals infected with SAD-B19ΔG-EnvA	1
Analysis of infected primary cells and animal tissues from non-human primates infected with SAD-B19ΔG-EnvA (as a class 1 activity)	2

If rabies viral vectors are modified to contain hazardous inserts, project leaders or biosafety officers can arrange additional personal protective measures such as the use of gloves, safety glasses and work in a biosafety cabinet (prevention of aerosols).

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8 Pseudorabies viral vectors

Pseudorabies Virus (PRV; equal synonym: Suid herpesvirus 1) is an enveloped DNA-virus with one linear double-stranded genome belonging to the family of *Alphaherpesviridae*.

In contrast to rabies virus, PRV is not a human-pathogen, despite its broad host range and its significant homology with Herpes simplex virus 1 and 2. It is pathogenic to pigs and causes Aujeszky's disease or pseudorabies. Also susceptible are cattle, sheep, goats, cats, dogs, rabbits, raccoons and laboratory animals such as rats and mice. Humans and non-human primates are not affected. In hosts, PRV is highly virulent and transmitted by air. Herds can be protected through immunization. In Switzerland, PRV is classified as group 2 organism and Aujeszky's disease is considered as an endemic disease to be eradicated.

The Bartha strain of PRV is an attenuated strain used as a vaccine for pigs. PRV Bartha is used for neuronal circuit tracing as it is less cytotoxic for animal cells and does not move bi-directionally, but only in a retrograde way. In contrast, PRV Bartha H129 strain appears to spread preferentially in the anterograde direction.

A genetically modified form of PRV Bartha is Ba2001. It is unable to replicate unless its genome is recombined by cre-recombinase. Another replication-deficient strain is strain PRV Δ IE180. IE180 protein codes for the master sole immediate early transcriptional activator of viral genes required for DNA replication and RNA transcription. Transcomplementation with IE180 can restore transient infectivity.

Envelope glycoprotein gD of PRV is essential for cell infection. PRV Δ gD mutants are replicated in infected cells, but progeny virions released by infected cells are not infectious anymore.

Even though considerable effort has been made to design replication-deficient PRV, it remains to be seen whether the use of replication-deficient PRV vectors for trans-synaptic tracing will increase in the future. According to the number of publications, the use of SAD-B19 Δ G is incomparably more frequent than the one of PRV, as for SAD-B19 Δ G tools have been developed for mono-synaptic tracing.

Classification of work with pseudorabies viral vectors

Vector/activity	Group/class
DNA plasmids	
Recombinant pseudorabies vector DNA plasmids or helper plasmids containing pseudorabies open reading frames in <i>E.coli</i> K12 or derivatives	1
Pseudorabies viral vector particles	
Production of replication-competent PRV Bartha vaccine strain or other attenuated replication-competent PRV on permissive cell lines of group 1	1
Infection of group 1 target cells or animals with replication-competent PRV Bartha or other attenuated PRV	1
Infection of group 2 target cells or animals with replication-competent PRV Bartha or other attenuated PRV	2
Production of replication-deficient PRV on permissive cell lines without helper virus of group 2	1
Infection of group 1 target cells or animals with replication-deficient PRV	1
Infection of group 2 target cells or animals with replication-deficient PRV	2

Vector/activity	Group/class
Pseudorabies viral vectors in animals or animal cells	
Animals which have been injected with replication-competent PRV Bartha vaccine strain or other attenuated replication-competent PRV into the brain (anterograde movement of PRV allows spread to peripheral neurons)	2
Analysis of infected primary cells and animal tissues infected with replication-competent PRV Bartha vaccine strain or other attenuated replication-competent PRV as long as there is still a chance of infectious PRV particles present	2
Animals which have been injected with replication-deficient PRV into the brain	1
Analysis of transduced primary cells or animal tissues with replication-deficient PRV	1

If pseudorabies viral vectors are modified to contain hazardous inserts, project leaders or biosafety officers can arrange additional personal protective measures such as the use of gloves, safety glasses and work in a biosafety cabinet (prevention of aerosols).

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<https://www.efbs.admin.ch/en/recommendations/recommendations-of-the-secb/>

9 Semliki forest virus (SFV) and Sindbis (SIN) virus expression system

Semliki forest virus and Sindbis virus belong to the *Togaviridae* (genus *Alphavirus*). They are widespread and need an insect vector for transmission. The enveloped viruses contain single-stranded positive RNA. The viruses are assigned to group 2 (see *Classification of organisms* <https://www.bafu.admin.ch/bafu/en/home/topics/biotechnology/publications-studies/publications/classification-of-organisms.html>).

Semliki forest virus and Sindbis virus expression systems consist of two components:

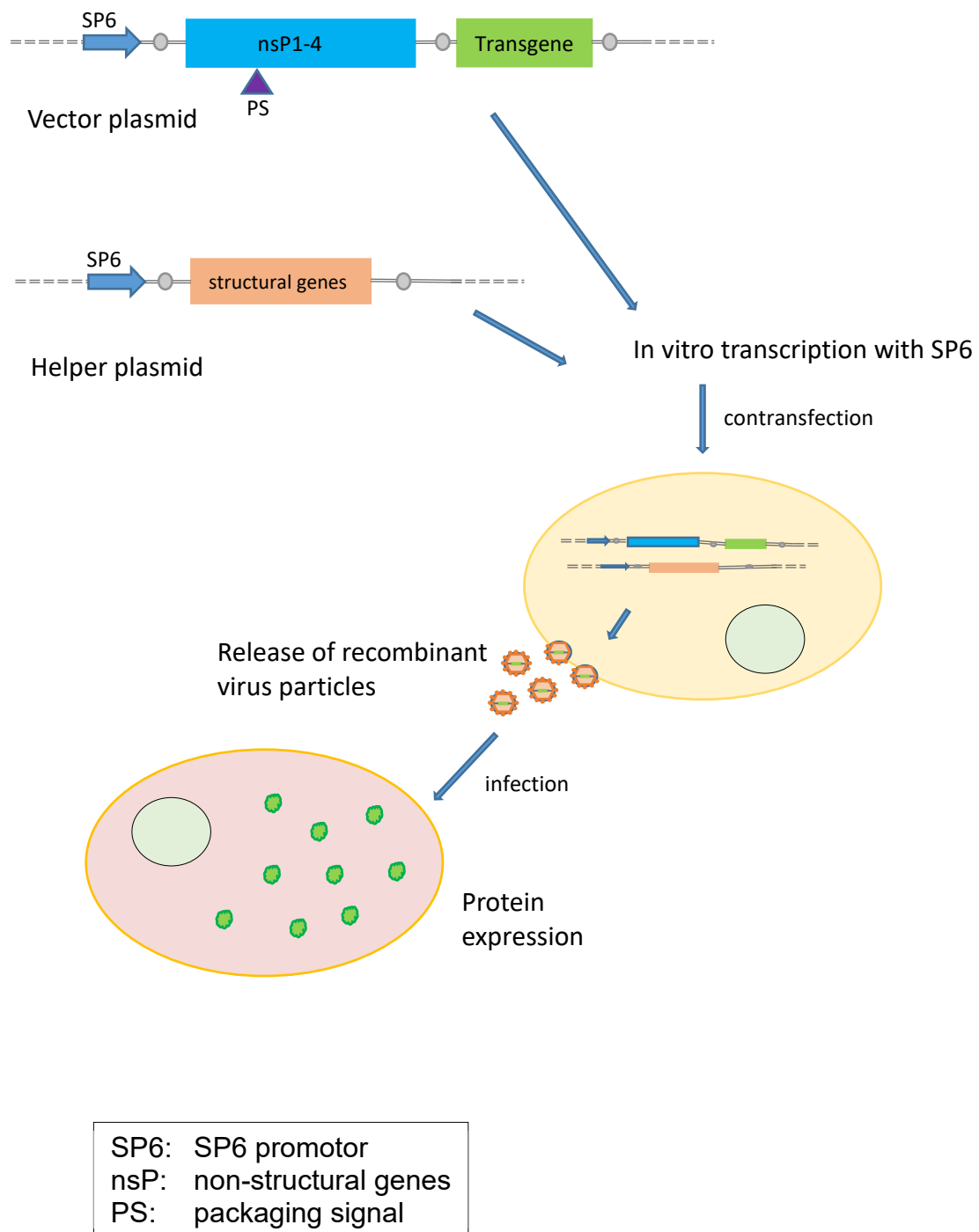
- The vector plasmid, consisting of a bacterial plasmid (a pBR322 derivative) and the viral non-structural genes under the control of the bacterial SP6 promoter, a packaging signal, and the transgene (replacing the viral structural proteins).
- The helper plasmid, consisting again of a bacterial plasmid (a pBR322 derivative) and the viral structural proteins under the control of the SP6 promoter. The helper plasmids lack the regions coding for the viral non-structural proteins and the signal for packaging of the viral RNA.

On one hand, the transgene can be expressed directly by transfecting the RNA transcribed *in vitro* from the expression vector (vector-RNA) into a eukaryotic cell. On the other hand, viral particles can be produced by co-transfecting eukaryotic cells with the *in vitro* transcribed RNAs from both the expression vector and the helper plasmid, whereby the vector-RNA will be packaged into viral particles by the structural proteins translated from the helper-RNA. Heterologous protein expression is eventually achieved by infecting eukaryotic cells with the recombinant viral particles (Figure 9.1). It is also possible that the sequences encoding the structural proteins are integrated into helper cell lines, where the vector-RNA is subsequently replicated and packaged into viral particles by the constitutively expressed viral structural proteins.

Alternatively, cDNAs coding for the genes for the non-structural viral proteins and the transgene on one plasmid and for the genes for the structural viral proteins on a second plasmid, are placed under the control of a Pol II-promoter. Unlike in the above described system, the vector- and helper-RNA are produced by the host cells following transfection with the plasmids.

Usually, replication-defective viral particles will be produced when using such vectors. The replication deficiency of the system is therefore a key element of risk assessment. However, when using a helper-plasmid with a complete and unmodified reading frame for the structural proteins, it cannot be excluded that infectious, replication-competent viruses are produced following co-transfection of eukaryotic cells with *in vitro* transcribed vector-RNA. Such an activity represents a low risk for humans and the environment.

Various systems exist in order to minimize or avoid the production of infectious, replication-competent SFV- or SIN-viruses. First efforts were obtained by introducing a triple mutation in the proteolytic cleaving site of the structural p62 component (dimeric E3-E2 precursor protein) in the helper plasmid, therefore reducing the probability of appearance of infectious and replication-competent virus through recombination of the various RNAs. The resulting viral particles may be replication-competent but are not infectious because uncleaved p62 has been integrated into the envelope. Viral particles can be made infectious by activation with α -chymotrypsin but will not be able to propagate in the infected cells (one-round-infection). Further developments have led to the distribution of the structural genes on two independent helper plasmids ("two-helper RNA system"), thus avoiding the production of complete infectious viral RNA through recombination events.



Adapted from ZKBS, 2017

Figure 9.1: Semliki forest virus (SFV) and Sindbis (SIN) virus expression systems

Classification of work with Semliki forest virus / Sindbis virus expression vectors

Vector/activity	Group/class
Expression vector or helper plasmid vector in <i>E. coli</i> K12 or derivatives	1
Transfection of group 1 eukaryotic cells with <i>in vitro</i> transcripts from SFV or SIN expression vectors, as long as the cells do not contain sequences coding for viral envelope proteins and that the heterologous protein has no hazard potential	1
Transfection of group 2 eukaryotic cells with <i>in vitro</i> transcripts from SFV or SIN expression vectors	2
Co-transfection of group 1 or group 2 eukaryotic cells with <i>in vitro</i> transcript of expression vector containing subgenomic viral or cellular sequences and transcript of helper-containing complete sequences for structural envelope proteins: infectious, replication-competent viral particles can be produced	2
Infection of group 1 or 2 eukaryotic cells with the above-mentioned viral particles	2
Co-transfection of group 1 or 2 eukaryotic cells with <i>in vitro</i> transcript of expression vector containing subgenomic viral or cellular sequences and transcripts of helper function derived from the "two-helper RNA system": only infectious, but replication-defective viral particles can be produced: <i>classification same as recipient organism</i>	1 or 2
Infection of group 1 or 2 eukaryotic cells with the above-mentioned viral particles: <i>classification same as recipient organism</i>	1 or 2
Co-transfection of group 1 or 2 eukaryotic cells with <i>in vitro</i> transcript of expression vector containing subgenomic viral or cellular sequences and transcript of helper with a triple mutation in the proteolytic cleaving site of the E3-E2 precursor protein: function derived from the "two-helper RNA system": replication-competent but not infectious viral particles are produced: <i>classification same as recipient organism</i>	1 or 2
Activation of the above-mentioned viral particle through α -chymotrypsin treatment (production of infectious viral particles that cannot propagate)	1
Infection of group 1 or 2 eukaryotic cells with the above-mentioned viral particles: <i>classification same as recipient organism</i>	1 or 2

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